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ORIGINAL ARTICLE

Androgen receptor polymorphism (CAG repeats) and androgenicity

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Summary

Objective Polymorphism of the androgen receptor (AR) has been related to various pathophysiological conditions, such as osteoporosis and infertility. The objectives of this study were to evaluate the frequency of distribution in a normal Italian population and to assess CAG repeats (CAGr) in other conditions, such as hypoandrogenism, potentially influenced by AR polymorphism.

Patients and measurements CAGr polymorphism was determined in a group of 91 healthy normoandrogenized subjects, 29 hypoandrogenized patients (hypoplasia of prostate and seminal vesicles, reduced beard or body hair, etc.) and 29 infertile patients by direct sequencing.

Results The mean (± SD) number of CAG repeats [(CAGr)n] was 21·5 (± 1·7) in the control group, 21·4 (± 2·0) in the infertile patients and 24·0 (± 2·9) in the hypoandrogenic males. The difference was statistically significant between this last group and the other two ($P < 0{\cdot}0001$), while there was no difference between normal controls and infertile patients. The frequency distribution showed a shift towards higher CAG length in hypoandrogenized patients compared to controls and infertile patients. If we used a cut-off point of 24·9 (2 SD above the mean), the percentage of patients with 25 or more CAGr repeats was 38% among hypoandrogenized patients, 7% among infertile patients and 5% among the control group. In hypoandrogenized subjects (CAGr)n correlated slightly with testis and prostate volume. The number of CAG repeats was not associated with any of the hormonal parameters, including testosterone, evaluated in the three groups.

Conclusions Our normal population, representing subjects from Central Italy, is superimposable on other European populations with regard to (CAGr)n distribution. Hypoandrogenic males have a shift in the frequency distribution towards longer (CAGr)n. Infertile patients are not statistically different from the control group. These findings suggest that, given the same amount of circulating testosterone, as in our hypoandrogenized and control group, the final net androgenic phenotypical effect is due to AR polymorphism.

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Introduction

Testosterone (T) effects are mediated through the androgen receptor (AR), a nuclear receptor belonging to the superfamily of steroid and thyroid hormone receptors.[1–3] This receptor is characterized by the presence of a variable number of glutamine residues in the NH_2-terminal domain. These residues are encoded by a variable number of CAG repeats [(CAGr)n] in the first exon of the gene. The (CAGr)n is inversely associated with the transcriptional activity of T target genes: that is, the higher the (CAGr)n, the lower the transcriptional activity and vice versa.[4,5] This started with the discovery that Kennedy's disease (a spinobulbar muscular atrophy) was characterized by a marked increase in (CAGr)n (beyond 37) and a hypoandrogenic status.[6] Following this report, the (CAGr)n has been analysed in a variety of cross-sectional studies, investigating its influence on clinical conditions and parameters affected by T action, such as bone density,[7] spermatogenesis,[8] mood variations, cognitive functions and pre-Alzheimer conditions,[9,10] and hair development in both men and women.[11] Recently, Zitzmann *et al.*[12] have correlated the prostate growth induced by T replacement therapy in hypogonadal men with (CAGr)n in a longitudinal pharmacogenetic study, demonstrating an impressive modulating effect by the CAGr polymorphism.

A role of the (CAGr)n has been hypothesized in determining the androgenicity of an individual.[12] This study was undertaken to evaluate the (CAGr)n in a series of patients with characteristics of hypoandrogenic traits and compare them with a group of normal controls and infertile patient.

Subjects and methods

Subjects

One hundred and forty-nine Italian (Caucasian race, mainly from Central Italy) subjects were included in this study. The control group comprised 91 normal fertile subjects without any history of gonadal disease and with normal bone mineral density and body hair distribution. The others ($n = 58$) were selected from patients referred to the andrology outpatient clinic of our department.

Twenty-nine were infertile according to this definition: 2 years of attempted conception in a stable couple relationship and at least one seminal parameter (concentration and/or motility) below World Health Organization (WHO) criteria.[13] They underwent semen analysis, scrotal ultrasound, prostate transrectal ultrasound whenever necessary, and hormonal assessment of FSH, LH, PRL and testosterone. In cases of severe oligospermia or azoospermia they underwent karyotyping and screening for microdeletions of the Y chromosome.

The other 29 were hypoandrogenized, with reduced facial or body hair, prostate or vesicular or epididymal hypoplasia, penile recurvatum. Body hair pattern was categorized by experienced clinicians according to the four-point scale proposed by Zitzmann *et al.*[14] (feminine = 1, scantly male = 2, normal male = 3, extraordinarily male = 4).

Among the patients of this last group none had testosterone plasma values below 8·67 nmol/l (2·5 ng/ml) and reduced testes volume. In those patients with a clinical suspicion of prostate or vesicular or epididymal hypoplasia this was confirmed by ultrasound, both scrotal and transrectal. In these same patients analysis of the CFTR gene was performed to exclude mutations or deletions.

Methods

Semen analysis

At least two semen analyses were performed according to WHO guidelines. Patients were considered infertile if the concentration fell $< 20 \times 10^6$/ml and progressive motility (classes $a + b$) < 50%. Sperm morphology was analysed, but not considered in this evaluation unless the sperm morphology was < 20%, as in our laboratory the percentage of normal forms in a fertile population was 25 ± 2·1%.

Ultrasound

Scrotal ultrasound was performed with a 7·5 MHz linear probe with a transducer length of 45 mm (to allow a precise evaluation of the longitudinal axis) (Esaote, Genua, Italy). To evaluate testis volume the ellipsoid formula ($\pi/6 \times a \times b \times c$, where a, b and c are the three axes of the testis) was used. Prostate and seminal vesicle evaluation was performed with an abdominal 3·5 MHz and a transrectal 7·5 MHz transducer: the formula used for assessment of prostate volume was the same as above. The ultrasound examinations were performed by one of the authors, highly experienced in the field.[15]

Hormone assays

Serum FSH, LH, T and PRL were measured by chemiluminescence (Beckman Coulter Access with Unicel™ DXI 800 Access Immunoassay System, Beckman Coulter). The intra-assay and interassay coefficients of variation (CVs) were, respectively, for FSH 3·3 and 5·0%; for LH 5·1 and 6·6%; for T 2·9 and 6·2% and for PRL 2·8 and 7·0%.

Molecular biology (genetic analyses)

Screening of microdeletions in the 5–6 interval of the Y chromosome was performed according to the European Academy of Andrology (EAA) and European Molecular Genetics Quality Network (EMQN) guidelines,[16] recently revised.[17] The following Y-specific sequence tagged sites were analysed: 84 and 86 (AZFa), 127 and 134 (AZFb), 254 and 255 (AZFc). As control, amplification of SRY (sY14) and ZFX/ZFY genes was performed.

Screening of the most common CFTR gene mutations ($n = 31$) was performed by multiple polymerase chain reaction (PCR).

Determination of (CAG)n

Genomic DNA was extracted from peripheral blood lymphocytes using a DNA blood mini kit (Quiagen, Oslo, Norway). The AR exon 1 region encoding the polyglutamine repeat was amplified using PCR. The primers used in the present study were AR1 (5′TGCGCGAAGTGATCCAGAAC3′) and AR2 (5′GCTGTGAAGGTTGCTGTTCCTCAT3′). PCR amplification was performed in a final volume of 50 µl containing 0·2–0·5 µg of DNA template, Taq Buffer 10× (Promega, Madison, WI, USA), $MgCl_2$ 25 mM (Promega), dNTP 10 mM (Promega) 1·25 units of Taq polymerase (Promega) and 50 pmol of each primer.

The thermal cycling profile on PTC200 (Peltier Thermal Cycler) consists of a 10-s denaturation step at 94 °C, a 45-s annealing step at the optimized temperature of 60 °C and a 20-s extension step at 72 °C for a total of 35 cycles. Each PCR was initiated with a 2-min denaturation step at 94 °C, terminating with a 10-s extension step at 72 °C and 4 °C permanently. The PCR-amplified products were loaded on agarose gel 1% for electrophoresis and purified with a Jetquick purification kit (Genomed, GmbH, Löhne, Germany).

The PCR-amplified products were subjected to direct sequencing, which was performed using a BigDye™ Terminator Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and a Genetic Analyser PRISM™ 310 (Applied Biosystems). All samples were screened by this method.

Informed consent

Informed consent was obtained from each patient or volunteer according to Italian legislation.

Statistical analysis

All variables were checked for normal distribution by the Kolmogorov–Smirnov one-sample test for goodness-of-fit. Analysis of variance (ANOVA) was used for intergroup comparison and when $P < 0{\cdot}05$ post-hoc tests were applied (Scheffe). Multiple regression was used for comparison between (CAGr)n and hormonal and clinical parameters.

Results

The main characteristics of the three groups are shown in Table 1. Confirming the selection criteria, body hair pattern was different in hypoandrogenized and control subjects. T and particularly FSH levels differed markedly between infertile patients and the other two groups; LH differed only between infertile and controls. This was probably due to the presence of patients with severe testicular damage among the infertile patients. The mean (± SD; SE) (CAGr)n

Table 1. Main characteristics of the three populations studied

		Infertile		Hypoandrogenized		Controls		*P*-value		
Parameter	Units	Range (mean ± SD)	No data	Range (mean ± SD)	No data	Range (mean ± SD)	No data	Hypo/Inf	Hypo/Contr	Inf/Contr
Age	year	22–47 (33·5 ± 6·9)	$n = 0$	18–52 (30·5 ± 8·3)	$n = 0$	23–45 (32·3 ± 6·3)	$n = 0$	0·15	0·31	0·65
Weight	kg	68–92 (77·7 ± 8·4)	$n = 0$	67–88 (76·6 ± 6·3)	$n = 0$	59–90 (75·8 ± 6·7)	$n = 49$	0·57	0·62	0·27
Body hair pattern*		(2·7 ± 0·57)	$n = 0$	(2·4 ± 0·73)	$n = 0$	(2·8 ± 0·57)	$n = 49$	0·10	0·02	0·94
FSH	U/l	0·8–30 (12·0 ± 8·4)	$n = 1$	1·4–18 (5·4 ± 4·8)	$n = 1$	1·3–9 (5·1 ± 2·4)	$n = 1$	< 0·0001	0·80	< 0·0001
LH	U/l	1·2–18·6 (5·6 ± 3·6)	$n = 1$	1·5–16 (4·2 ± 3·1)	$n = 1$	1·3–7·8 (4·2 ± 1·7)	$n = 1$	0·07	0·99	0·04
PRL	µg/l	96–302 (166 ± 44)	$n = 1$	70–438 (208 ± 86)	$n = 1$	62–366 (188 ± 58)	$n = 1$	0·10	0·19	0·59
T	nmol/l	4·2–19·4 (12·1 ± 3·8)	$n = 1$	8·7–34·7 (15·3 ± 7·3)	$n = 1$	10·1–24·6 (15·3 ± 3·8)	$n = 1$	0·02	0·89	0·01
Bitesticular volume	ml	1·4–38·3 (20·2 ± 9·4)	$n = 2$	27·0–42·8 (32·0 ± 4·1)	$n = 2$	25–43 (34·8 ± 4·3)	$n = 40$	< 0·0001	0·17	< 0·0001
Prostate volume	ml	18–28 (23·4 ± 2·8)	$n = 8$	13–28 (21·1 ± 4·6)	$n = 9$	19–31 (24·1 ± 2·6)	$n = 45$	0·04	0·003	0·49
CAGn length	*n*	18–26 (21·4 ± 2·0)	$n = 0$	19–30 (24·0 ± 2·9)	$n = 0$	18–26 (21·5 ± 1·7)	$n = 0$	< 0·0001	< 0·0001	0·92

*Four-point body hair scale: 1 = feminine; 2 = scantly male; 3 = normally male; 4 = extraordinarily male.
Hypo, hypoandrogenized; Inf, infertile; Contr, controls.
Intergroup comparison performed with ANOVA and then post-hoc tests.

was 21·5 (± 1·7; 0·1) in the control group, 21·4 (± 2·0; 0·3) in the infertile patients and 24·0 (± 2·9; 0·5) in the hypoandrogenic males. The difference was statistically significant between this last group and the other two ($P < 0·0001$), while there was no difference between normal controls and infertile patients. The frequency distribution shows a significant trend in hypoandrogenized patients towards higher (CAGr) length (Fig. 1). The 90th percentiles in hypoandrogenized, infertile and control subjects were 29·0, 24·0 and 23·4, respectively. If we used a cut-off point of 24·9 (2 SD above the mean), the percentage of patients with 25 or more (CAGr)n among hypoandrogenic men was 38%, among infertile patients 7% and among the control group 5%.

The (CAGr)n did not correlate with any of the hormonal parameters evaluated (FSH, LH, T and PRL) in any of the three groups (Table 2). Specifically, the (CAGr) length was not associated with T serum levels in the hypoandrogenic males (Fig. 2). In the hypoandrogenized group an inverse correlation between (CAGr)n and testis ($P = 0·04$) and prostate ($P = 0·05$) volume was found at the limit of significance.

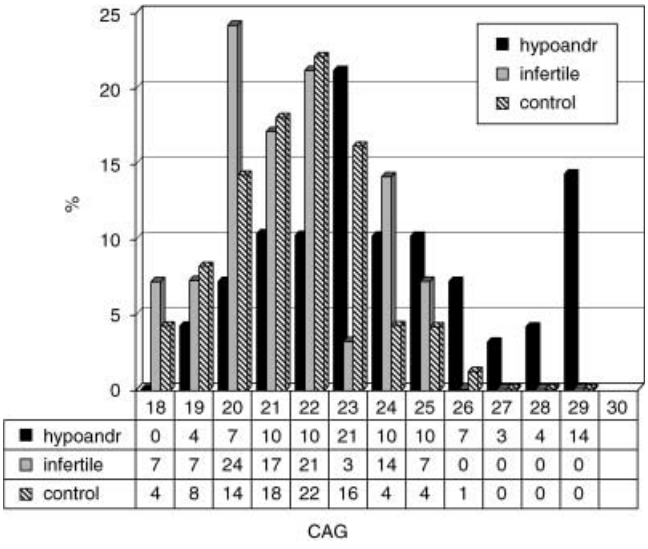

Fig. 1 Frequency distribution of number of CAG repeats among 91 normal controls, 29 hypoandrogenic males and 29 infertile patients. A shift towards longer CAG repeats is seen in hypoandrogenized patients compared to infertile men and the control group.

Discussion

Various recent reports have stressed the importance of AR polymorphism and particularly the (CAGr)n, in determining different pathophysiological conditions, such as bone metabolism, prostate development and cancer, hair development, and neurological disorders.[18] The AR operates as a ligand-inducible transcription factor that mediates the expression of target genes in response to specific ligands. The NH_2-terminal domain is the most variable among nuclear receptors in terms of both length and sequence. There are two discrete regions within the NH_2-terminus that contribute to transactivation. The full-length receptor requires a region primarily

Table 2. Correlation between length of CAG repeats and clinical, hormonal and ultrasound parameters (multiple regression)

		Infertile CAGn length		Hypoandrogenized CAGn length		Control CAGn length	
Variable	Units	Std coeff.	*P*	Std coeff.	*P*	Std coeff.	*P*
Age	years	0·11	0·63	−0·1	0·62	−0·09	0·6
FSH	U/l	−0·12	0·69	−0·06	0·84	−0·04	0·9
LH	U/l	0·12	0·9	0·25	0·43	−0·08	0·7
PRL	µg/l	−0·23	0·25	−0·14	0·55	0·16	0·27
T	nmol/l	−0·09	0·81	−0·09	0·63	−0·2	0·25
Bitesticular volume	ml	−0·31	0·24	−0·5	0·04	0·28	0·07
Prostate volume	ml	−0·36	0·10	−0·44	0·05	0·17	0·2

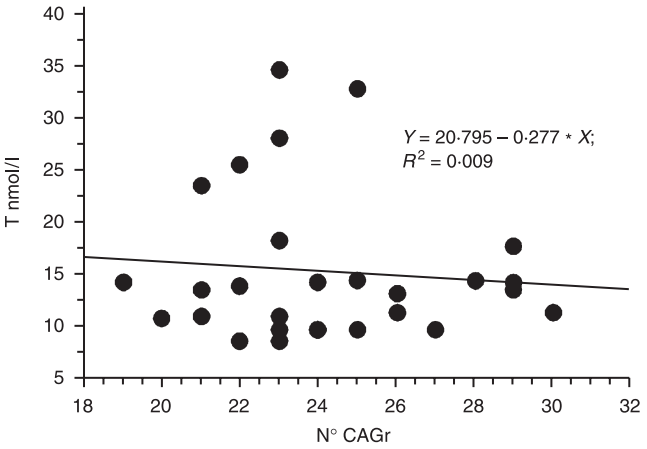

Fig. 2 Testosterone levels in relation to CAGr length of the AR gene of hypoandrogenized patients. Simple regression is given ($P = 0{\cdot}63$).

located between amino acids 141 and 338 for full ligand-inducible transcriptional activity.[19,20] This region contains a polymorphic polyglutamine repeat that ranges from 8 to 31 repeats in normal individuals, with a modal length of 20.[21,22] Longer polyglutamine tract length results in decreased AR transcriptional activity *in vitro*.[23,24]

The biological substrate of these correlations is represented by the modifications of binding with coactivators, transcription factors and corepressors determined by CAGr length. The polyglutamine tract forms part of the interaction surface for the AR coactivator ARA24 and expansion of the polyglutamine tract from 25 to 49 repeats results in a reduction of AR–ARA24 interactions, possibly because the expanded glutamine repeats result in an abnormal conformation of the AR NH_2-terminal.[25,26]

The mean, median and frequency distribution among our normal population was superimposable on the series of Harkonen *et al.*[27] in Finnish men, although our range was narrower.[18–26] Therefore, we have shown that the Italian population, in this case of Central Italy, is comparable to other European populations, at least concerning this specific genetic aspect.[28,29]

Our data demonstrate for the first time that hypoandrogenic traits, such as hypoplasia of prostate and seminal vesicles, and reduced beard and body hair, are characterized by a shift in the frequency distribution of (CAGr)n towards a prevalence of longer repeats. It might be that, given the same amount of circulating T, as in our hypoandrogenized and control groups, the final net androgenic phenotypical effect is due to the AR polymorphism. This would support the hypothesis that it is not the ligand, that is T, that determines androgenicity, but the interrelation between the polyglutamine surface of AR and coactivators, such as ARA24.[26] The work by La Spada *et al.*[6] showed the extreme of this pattern with very significant hypoandrogenic traits associated with very long (CAGr)n as those found in patients with Kennedy's syndrome. Similar findings were recently described in a very elegant study by Zitzmann *et al.*[14] in Klinefelter patients. In these patients longer CAG repeats were associated with lower bone density, increased height, gynaecomastia, lower professional achievement and a lower tendency to form a stable couple relationship. The inverse correlation between (CAGr)n and testis volume found by these authors was partially confirmed by our data. A possible explanation for this finding is that, given sufficient gonadotrophic stimulation and normal Leydig cell responsiveness, T action on the testis itself, as for any other target organ, is mediated by AR polymorphism in the long term, as hypothesized previously by Zitzmann *et al.*[14] Another specific target of androgen action, that is the prostate (volume), shows a significant difference between hypoandrogenized patients and controls. As this difference cannot be ascribed to a variance in T levels, is most probably related to AR polymorphism. The lower (compared to testis volume) correlation between (CAGr)n and prostate volume in hypoandrogenized subjects is probably due to the lower number of cases analysed and the wider range.

Concerning the infertile population, even if this was not the aim of our work, our data, in a reduced number of patients, support the hypothesis that this clinical condition does not correlate with the length of (CAGr)n, as shown previously by Rajpert-De Meyts *et al.*[30] and Thangaraj *et al.*[31] and in disagreement with the findings of Tut *et al.*[32] In fact our infertile population shows a broad frequency distribution (see Fig. 1), with peaks at 20 and 24–25 CAG repeats, demonstrating the heterogeneity of this population. It is likely that within this group of patients single cases could be related to polymorphism of the AR, but the latter does not seem to be a major determinant of male infertility.

Prospectively, the assessment of (CAGr)n in every patient with an andrological disease could provide some indication of the pathophysiological causes of that condition. As shown by Zitzmann *et al.*[12]

in their work on hypogonadal patients and the correlation between (CAGr)n and prostate volume, a preliminary assessment of (CAGr)n could guide therapeutic modalities (the frequency and amount of exogenous androgens). For example, prescribing exogenous T in a patient with a serum T level in the lower normal range or slightly reduced, but with (CAGr)n in the lower end of the curve, could be of no benefit for the parameter the clinician would like to treat, such as bone density or erectile dysfunction, and could even be dangerous at the prostatic level. Conversely, as suggested by Zitzmann *et al.*,[14] early treatment of Klinefelter patients with longer (CAGr)n could help them to achieve an adequate androgenicity during a particular period of life, such as pubertal development, when some physical and psychological parameters are heavily influenced by the androgenic milieu.

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